Kinetics of the Carbamylation of Cholinesterase

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SUMMARY

The kinetics of inhibition of acetylcholinesterase by three methylcarbamates was explored, and it was shown that the observations of both short-term and steady-state inhibitions were satisfactorily accounted for if one assumed that a reversible complex was formed prior to carbamylation of the enzyme. New kinetic evidence for the existence of such a complex was also provided. Both the complex formation and the carbamylation steps were inhibited by the substrates acetylthiocholine and 1-naphthyl acetate. The decarbamylation step was not affected by 2-pyridine-aldoxime methiodide. 1-Naphthyl methylcarbamate did not affect a muscle preparation containing acetylcholine receptor.

INTRODUCTION

The precise mechanism by which the carbamates inhibit cholinesterase has been a subject of some controversy, particularly as to whether a simple reversible complex is formed (Eq. 1).

$$E + CX \rightleftharpoons ECX \qquad (1)$$

where E is the enzyme, C the group $C(O)NR_2$, and X the remainder of the carbamate; or whether carbamylation occurs, with the *overall* reaction (Eq. 2):

$$E + CX \rightarrow EC + X \qquad (2)$$

Equation 2, suggested by Goldstein (1) in 1951 was confirmed by the demonstration by Wilson *et al.* (2, 3) that enzyme which has been inhibited by carbamates recovers from inhibition at a rate dependent on the nature of C, and independent of the nature of X, implying that X must have left in the course of inhibition, and that the recovery takes the form (Eq. 3):

$$EC \rightarrow E + C \qquad (3)$$

Additional evidence was the demonstration by O'Brien *et al.* (4) that inhibition was accompanied by release of X at the predicted rate, in accordance with the overall reaction of Eq. 2. They also showed that the kinetics of inhibition were in accordance with an equation of a form identical with that developed by Kitz and Wilson (5) in 1962 for inhibition by methanesulfanate inhibitors and by Main (6) in 1964 for inhibition by organophosphates. This equation is based on the formation of a reversible complex, ECX, followed by carbamylation (k_2 step) and subsequent recovery by decarbamylation (k_3 step):

$$E + CX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ECX \overset{k_2}{\underset{X}{\rightarrow}} EC \xrightarrow{k_3} E + C \qquad (4)$$

The concentrations of the above species at any given time will be shown as (E), (CX), etc., and the initial concentration of E as (E_0). The value k_{-1}/k_1 is also termed K_a, the dissociation constant of ECX. When conditions are such that k_3, which is the slowest step, can be neglected (i.e., when the experiment is performed within a few minutes) it was shown [O'Brien *et al.* (4)] that the equation derived from the above formulation is indeed followed:

$$\frac{1}{i} = \frac{t}{2.3\Delta \log V} \cdot \frac{k_2}{K_a} - \frac{1}{K_a} \qquad (5)$$

where i is the inhibitor concentration, t the time of incubation (the term t is used throughout in place of the Δt of the original formulation), and $\Delta \log V$ the change, caused by inhibition, in the logarithm of the velocity of the reaction with substrate. Equation 5, which we shall call the Main equation because we have employed his form of it, was used by O'Brien *et al.* (4) to measure approximate values of K_a and k_2 from data describing the dependence of $\Delta \log V$ upon i. Conformity with the Main equation was the only evidence that in fact a reversible-complex step was involved.

The present study has three principal objectives. One is to examine the kinetics of inhibition which prevail when k_3 cannot be neglected, i.e., in prolonged experiments, where steady-state conditions prevail; a second is to reexamine the validity of the Main equation with respect to short-term carbamate inhibition; and a third is to provide quite different, independent evidence that a reversible-complex step does occur.

Much of the work was performed with a purified sample of the insecticidal carbamate called carbaryl. It is 1-naphthyl methylcarbamate.

METHODS

Winthrop bovine erythrocyte acetylcholinesterase (AChE) was used for all tests. All reagents were made up in phosphate buffer 0.05 M and pH 7.0. A water bath attachment was employed so that a constant temperature of 38° was maintained during the tests. The Main kinetics were performed by the acetylthiocholine method previously described (4), but with the time of incubation with inhibitor reduced to 20 sec. This permitted the use of higher inhibitor concentrations. The zero-time studies were performed as follows.

Modified Ellman procedure. Measurements were made with a Beckman DU Spectrophotometer at 412 mμ using the color reagent 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (7). The machine was zeroed on a blank containing all the reagents except AChE. Acetylthiocholine was made up at 3.75×10^{-4} M in buffer containing 1 mg DTNB per milliliter. The final dilution of the carbamates was made with this solution. Additions of reagents during the tests were made with a syringe to achieve rapid mixing.

AChE, 1.5 ml, (1 unit/ml), was pipetted into the cuvette. At zero time, 1 ml of acetylthiocholine was added and readings were taken every 10 sec for 1 min. Then 0.5 ml of acetylthiocholine was added, and readings again were taken every 10 sec to establish the change in the rate of the reaction due to dilution. The procedure was repeated using 0.5 ml of carbamate solution (containing acetylthiocholine also) for the final addition, and readings were taken every 10 sec until no further change in the absorbance was observed.

1-Naphthyl acetate method. Measurements were made with an Aminco-Bowman spectrophotofluorometer activating at 300 mμ and emitting at 455 mμ (the peak for 1-naphthol); maximum fluorescence of 1-naphthyl acetate was found at 340 mμ. Glass-distilled water was used for all preparations. 1-Naphthyl acetate was made up at 4×10^{-4} M. The procedure was similar to the modified Ellman method except that the initial volume of AChE was 1 ml. A separate set of readings was taken to establish the rate of spontaneous hydrolysis of 1-naphthyl acetate, and these were subtracted from the test readings.

Reactivation. Two problems were encountered in reactivation attempts with pyridine 2-aldoxime methiodide (2-PAM). High concentrations inhibited the AChE. A 2-PAM final concentration of 10^{-4} M was therefore used. This concentration interfered with the ASCh method which was then being used, and therefore the following procedure was used. Dry AChE, 125 units, was added to 0.25 ml of carbaryl, 5×10^{-6} M in buffer, and incubated at 38° for 15 min. Then 250 ml of 2-PAM, 10^{-4} M in buffer was added, the solution was held at 38°, and samples of 0.4 ml were withdrawn at intervals and added to 0.4 ml of acetylcholine 8×10^{-3} M in water, held at 38° for 3 min; residual acetylcholine was then measured by the method of Hestrin (8).

Frog rectus preparation. The frog rectus

abdominis muscle was used with a Ringer solution at room temperature (9). A good linear relation was found between contraction and acetylcholine concentrations of 1–10 μM. In order to study carbamate effects at the receptor level only, it was necessary to destroy AChE. This was done by incubating with 10 μM paraxon for 20 min, then washing twice. (Prior experiments established that this procedure destroyed all the AChE, as judged by the fact that additional incubations led to no further sensitization.) The response was now 2-fold more sensitive than before; it was measured with 0.5 μM acetylcholine. The effect of carbaryl was measured by incubating with 100 μM carbaryl for 15 min, then washing and measuring again with 0.5 μM acetylcholine.

RESULTS

Steady-State Inhibition

The Main equation is only applicable when the inhibition time is short enough that the k_3 step can be ignored. With long periods of incubation of enzyme with inhibitor, it has been observed (4, 10, 13) that for any given concentration of carbamate i, the plot of residual enzyme activity against time diminishes initially in a first-order way (as in Fig. 1) and then forms a plateau whose height is characteristic of i.

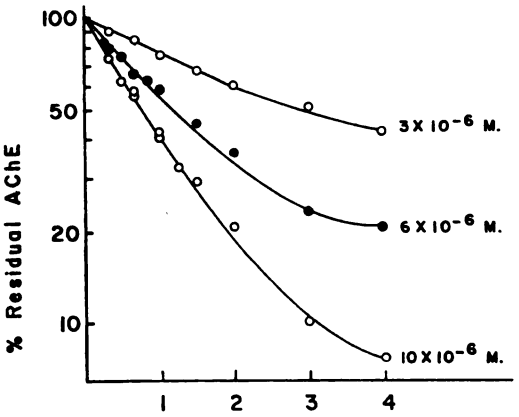

FIG. 1. *Progressive inhibition of acetylcholinesterase by various concentrations of carbaryl, with substrate added after the indicated times (minutes)*

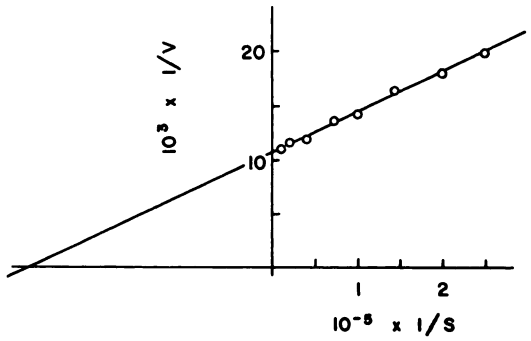

FIG. 2. *Relation between carbaryl concentration (S) and steady-state percent inhibition (V) of acetylcholinesterase*

Substrate (acetylthiocholine, 2.5 mM final concentration) added after incubation with inhibitor. Subsequent assay as in O'Brien *et al.* (4).

The question is, what parameters determine the plateau height?

Equation 4 makes it clear that carbamates are substrates for cholinesterase; it is because of the slow k_3 step, whose half-life is 19 min at 38° for methylcarbamates [O'Brien *et al.* (4)], that they have a slow enough turnover to appear as inhibitors. The plateau heights measure the amount of enzyme still uncarbamylated; thus a plateau at 60% of initial enzyme activity means[1] that 60% is uncarbamylated, 40% is carbamylated, i.e., is in the form EC. The velocity of the overall reaction with carbamate is given by k_3 (EC), because k_3 is the slowest step (the k_2 step has a half-life in the order of 0.5 min). The relative velocity (V) is thus directly proportional to (EC), which, when expressed as a percentage of (E_0), is the percent inhibition. Consequently, using percent inhibition as a measure of V, one can plot $1/V$ against $1/S$ (S in this case being the carbamate concentration) in the usual Lineweaver-Burk plot, and hence evaluate K_m for the reaction of Eq. 4. Such a plot is shown in Fig. 2 for carbaryl. By this procedure, the K_m values for three carbamates were evaluated, and are shown in Table 1.

[1] Under assay conditions such that the large excess of acetylcholine used succeeds in displacing that carbamate which is bound to the enzyme as reversible complex, ECX.

TABLE 1

Evaluation of constants by Main analysis and Lineweaver-Burk analysis

Standard errors are given after each value. Main and Iverson (19) have pointed out statistical advantages in plotting $it/2.3\Delta \log V$ as a function of i, instead of the original procedure of plotting $t/2.3\Delta \log V$ as a function of $1/i$. This procedure yielded for carbaryl figures of $10.9 \pm 44 \times 10^{-4}$M for K_a and 12.1 ± 0.69 min^{-1} for k_2, in excellent agreement with the findings in the table.

Main analysis was performed with incubation times of 20 sec. This time, being well within the first-order part of the inhibition (Fig. 2) gives better values than our earlier approximations [O'Brien *et al.* (4)].

	Carbaryl	3,5-Diisopropylphenyl methylcarbamate	2-Isopropoxyphenyl methylcarbamate
From Main analysis			
k_2	12.2 ± 0.67	—[a]	6.07 ± 0.61
$10^5 \times K_y$	10.1 ± 1.84	—[a]	4.67 ± 0.67
From Lineweaver-Burk analysis			
$10^7 \times K_m$	3.47 ± 0.37	0.544 ± 0.015	2.99 ± 0.28
$10^{-5} \times k_i$	0.825 ± 0.036	4.94 ± 0.39	1.21 ± 0.076
$10^5 \times K_y$	0.19	2.8×10^{-2}	2.0
k_2	0.16	0.15	2.44

[a] For this compound, the line extrapolated from the experimental points appeared to go through the origin, so that Main kinetics could not be used. Solubility limitations prevented the use of high concentrations, which give points close to the axis.

In Fig. 2, the intercept on the y axis is greater than 10×10^{-3}, i.e., even infinite carbamate concentration does not give 100% inhibition. The reason is as follows. The rate of formation of EC is k_2(ECX), and of its breakdown is k_3(EC); at steady state (ECX)/(EC) = k_3/k_2. Using $k_3 = 0.037$ [O'Brien *et al.* (4)] and k_2 for carbaryl = 12.2 (Table 1), then (ECX)/(EC) = 0.003. Thus with saturating carbaryl concentrations, 0.3% of the enzyme is in the form (ECX), which under the assay conditions of Fig. 2 appears as uninhibited enzyme.

Let us now consider what the value of K_m indicates about the constants of Eq. 4. Gutfreund and Hammond (11) have considered the precisely analogous case of hydrolysis of substrates by chymotrypsin. They have shown that, in the terms used herein:

$$K_m = \frac{k_3}{k_2 + k_3} \cdot \frac{k_{-1} + k_2}{k_1} \qquad (6)$$

The steady-state inhibition (V) caused by a given carbamate concentration (S) could therefore be evaluated if all the constants in Eq. 6 were known. It will be shown below that the value $(k_{-1} + k_2)/k_1$ is readily obtained; k_2 can be obtained by Main analysis (4) and k_3 by studying reactivation of inhibited enzyme (2–4).

Short-Term Inhibition

Having explored the kinetics of long-term (steady-state) inhibition, certain new facts become available to enable a fresh examination of the problem of short-term inhibition, which was studied in a previous paper (4). The Main equation, Eq. 5, describes events occurring in the short term. In the development of Eq. 5, Main used an argument (which will here be translated into terms of carbamate inhibition) analogous to that familiar in Michaelis-Menten kinetics, that under steady-state conditions

$$\frac{(E)(CX)}{(ECX)} = K_a \qquad (7)$$

As in the Briggs-Haldane correction of the Michaelis-Menten treatment, Eq. 7 is true only when k_{-1} is much larger than k_2. It is based on the approximation that the principal rate of breakdown of ECX is described by

$$-\frac{d(ECX)}{dt} = k_{-1}(ECX) \qquad (8)$$

However, this relation is correct only if k_2 is much smaller than k_{-1}. If such is not the case, the correct relation is:

$$-\frac{d(ECX)}{dt} = (k_{-1} + k_2)(ECX) \quad (9)$$

When this consideration is inserted into Main's argument, the result is not Eq. 5, but:

$$\frac{1}{i} = \frac{t}{2.3\Delta \log V} \cdot \frac{k_2 k_1}{k_{-1} + k_2} - \frac{k_1}{k_{-1} + k_2} \quad (10)$$

Equation 10 shows that the precise value given by the reciprocal of the y intercept of a Main plot (i.e., a plot of $1/i$ against $t/2.3\ \Delta \log V$) is not k_{-1}/k_1 which is K_a, but rather $(k_{-1} + k_2)/k_1$, which we may refer to as K_y. In the case that k_2 is much smaller than k_{-1}, K_y is almost identical to K_a.

It follows that the values which we previously reported (4) for K_a, which were determined by Main analysis, should more correctly be called K_y. Whether K_y is a close approximation of K_a depends upon determination of the relative sizes of k_{-1} and k_2, a matter examined under Discussion.

The introduction of the parameter K_y suggests a reexamination of Eq. 6, which may be rewritten:

$$K_m = \frac{k_3 K_y}{k_2 + k_3} \quad (11)$$

With this equation one may check the accuracy of any one of the values for these constants. For example, one may compute a value for k_3 and compare it with experimental measurements. For carbaryl, using values of K_y and k_2 from Main analysis, and K_m from Lineweaver-Burk analysis (Table 1), one may calculate from Eq. 11 that $k_3 = 0.042$ min^{-1}. This finding compares favorably with values of 0.055 min^{-1} from leaving-group analysis and 0.037 min^{-1} from recovery times on dilution (4).

Attempts to Simplify the Measurement of K_y

In view of the fact that Main analysis provides a value for K_y rather than the much-desired K_a (desired because K_a is a reciprocal measure of affinity), one might elect to find this less desirable value by a less laborious technique. A family of curves showing, for various inhibitor concentration, the logarithm of fractional activity as a function of time can yield K_m (by plotting the plateau heights in Lineweaver-Burk plots) and also k_i [the apparent first-order rate constant; $k_i = k_2 K_y$ (refs. 5, 6)] because a value of k_i is given by each of the first-order portions of the curve, whose slope is $-k_i\ i/2.303$ (ref. 12). Equation 11 can be rearranged, and the considerations inserted that $k_i = k_2/K_y$ whence:

$$K_y = \frac{1}{\frac{1}{K_m} - \frac{k_i}{k_3}} \quad (12)$$

Thus by using the value $k_3 = 0.037$ min^{-1} obtained from recovery experiments, one can obtain K_y and therefore k_2. Unfortunately, the problem arises of a small difference between very large numbers in the denominator.

The results for three carbamates are given in Table 1 under the heading, "From Lineweaver-Burk" analysis, but the agreement with the values obtained by Main analysis is poor, and therefore the procedure is not recommended.

An Independent Proof of Complex Formation

Doubts have been expressed by Reiner and Simeon-Rudolf (13) about the existence of a reversible complex (see Discussion). In response to these doubts, it is desirable to give an independent proof of complex formation. The only evidence for complex formation described so far (4) has been conformity with Main kinetics, especially in a positive intercept on a Main plot, which implies that at short incubation times, even infinite carbamate concentrations do not give infinite inhibition, i.e., total conversion of enzyme to EC. There is therefore a saturation phenomenon analogous to that observed with conventional substrates; once the enzyme is saturated with carbamate, the rate of formation of EC (which is the only species measured as "inhibited enzyme" when one works with

low concentration of carbamate and high concentration of acetylcholine for assay) cannot be increased by increasing the concentration of carbamate.

An alternative approach would be to examine continuously a reaction of enzyme with substrate, then at zero time, add in carbamate at a concentration large enough to compete with the substrate and give, virtually instantly, a significant amount of reversible complex, ECS. After this prompt reaction, one should then observe the progressive carbamylation reaction, its rate being a measure of k_2. In short-term experiments, the contribution of k_3 would be negligible.

Studies were therefore performed using either of two substrates (acetylthiocholine or 1-naphthyl acetate) each by a different assay procedure, and three different carbamates. The techniques used permitted continuous monitoring of the reaction rate. The substrates were used at a concentration equal to their K_m's, and the inhibitors at concentrations high enough to give complete inhibition in 3 minutes. Enzyme and substrate at 38° were mixed, allowed to react for 1 minute to establish the rate of hydrolysis, then (at "zero time") carbamate was added. Figure 3 shows that at zero time there was indeed substantial inhibition, as shown by extrapolation back to zero time and correction for the dilution involved in adding carbamate.

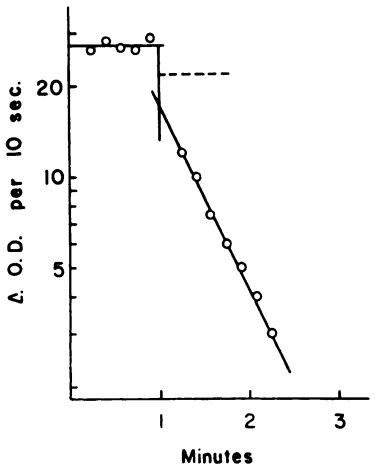

FIG. 3. *Rate of hydrolysis of 2.5 ml of 1.5 × 10^{-4} M acetylthiocholine by AChE*

At the vertical ("zero time") 0.5 ml of 2-isopropoxyphenyl methylcarbamate (5×10^{-5} M final concentration containing also 1.5×10^{-4} M acetylthiocholine) was added. Dashed line shows reduction in hydrolysis caused by addition of 0.5 ml of acetylthiocholine alone. "Minutes" refers to the time after acetylthiocholine was added; for time of incubation of carbamate plus enzyme, deduct 1 min.

Figure 3 therefore demonstrates that under appropriate circumstances (selected so that the carbamate concentration is high enough that measurable complex can exist even when substrate is present) one can see two kinds of inhibition reaction: one is very fast, occurring immediately after adding carbamate; the second is much slower. These two reactions we interpret as being due to rapid formation of complex ECX, and slower conversion of ECX to EC (with concomitant replenishment of ECX from E plus CX).

The intercept at zero time was calculated by a computerized regression procedure. For each carbamate except carbaryl, at least two runs with each of the two substrates methods were performed; each run employed a different solution of enzyme and a different stock solution of carbamate. In the case of carbaryl, the 1-naphthyl acetate method could not be used because of the fluorescence of the carbaryl. At zero time, the inhibition can be regarded as of a simple competitive type, and therefore [see Webb (14)] described by:

$$V = V_m \frac{(S)}{(S) + K_s\left(1 + \frac{i}{K_v}\right)} \qquad (13)$$

Where (S) is the substrate and i the inhibitor concentration, and V is the velocity of the substrate reaction just after addition of inhibitor. The value employed for V_m was twice the uninhibited substrate velocity, because the reaction was performed with a substrate concentration equal to K_m (the true V_m cannot be experimentally measured because of excess substrate inhibition). For K_s in Eq. 13, which is the dissociation constant for substrate and en-

TABLE 2

Estimates of K_y from zero-time inhibition

The K_m values used (as approximations of K_s) in the calculation of K_y from Eq. 13 were experimentally determined, the values being 1.45×10^{-4} M for acetylthiocholine and 1.91×10^{-4} M for 1-naphthyl acetate at 38° and pH 7.0.

	Acetylthiocholine method			1-Naphthyl acetate method			
	$10^5 \times$ carbamate conc.	Zero time inhibition	$10^5 \times K_y$	$10^5 \times$ carbamate conc.	Zero time inhibition	$10^5 \times K_y$	$10^5 \times$ mean K_y
Carbaryl	5	19	11.1	—	—	—	12.8
	5	14	15.6	—	—	—	—
	5	18	11.6	—	—	—	—
o-Isopropoxyphenyl methylcarbamate	5	26	7.1	5	27	6.5	7.9
	5	21	9.4	5	23	8.5	—
3,5-Diisopropylphenyl methylcarbamate	0.5	6	3.6	1	14	3.0	3.0
	0.42	8	2.5	0.72	11	3.0	—
	0.6	10	2.7	—	—	—	—

zyme, the K_m for the appropriate substrate was used as the nearest available approximation.

Calculations of K_y for three carbamates could therefore be made. They are shown in Table 2. Clearly there is good agreement between runs and between different substrates. Furthermore, agreement with K_y calculated from Main analysis (Table 1) is very good.

Substrate Inhibition of the k_2 Step

The data of Fig. 3 have been shown to give values of K_y determined by the zero-time method, which are in good agreement with K_y determined by Main analysis. It should also be possible to obtain a value for k_2, which should be given directly by the slope of the progressive part of the inhibition reaction. Examination of Fig. 3 shows a half-life of 36 sec for the progressive part, implying a k_2 of 1.15 min^{-1}. This is about one-tenth of the k_2 calculated by Main kinetics (Table 1). The most crucial difference between the zero-time and Main methods is that in the former, substrate (1-naphthyl acetate or acetylthiocholine) is present at all times, and the concentrations of substrate and inhibitor are such as to permit inhibition to continue in the presence of substrate. The inhibitions reported therefore occur in the presence of substrate. In the Main method, more substrate (acetylcholine) and less inhibitor is used, so that the inhibition reaction stops when substrate is added. Consequently the inhibitions reported occur in the absence of substrate.

It therefore appears that the k_2 step proceeds far more slowly when substrate is present, i.e., it is inhibited by substrate. Table 3 shows that the extent of this inhibition varies a good deal with carbamate and with substrate, presumably reflecting variations in the relative affinities of substrates and carbamates for some group essential in the actual carbamylation step. It should be emphasized that this phenomenon occurs quite independently of the usual competition for binding (i.e., competition at the K_a step).

The phenomenon of inhibition of the k_2 step was independently demonstrated by an experiment originally designed to confirm that simple competition (i.e., of the K_a step) occurs in a predictable way. Acetylthiocholine at a variety of concentrations (such that K_m/S varied from 2.8 to 0.07) was mixed with carbaryl at a concentration 2 K_y, and added to the enzyme. Twenty seconds later, the reaction was stopped and the amount of substrate hydrolysis meas-

TABLE 3
Inhibition of the k_2 step by substrates

The "k_2 without substrate" is from Main kinetics, Table 1, except for diisopropylphenyl methylcarbamate, for which it was necessary to compute it from $k_2 = k_i K_v$, using k_i from Table 1 and K_v from Table 2.

Carbamate	k_2 without substrate	1-Naphthyl acetate k_2	1-Naphthyl acetate % inhibition	Acetylthiocholine k_2	Acetylthiocholine % inhibition
Carbaryl	12.2	—	—	1.15	91
o-Isopropoxyphenyl methylcarbamate	6.07	1.59	74	0.49	92
3,5-Diisopropylphenyl methylcarbamate	3.63	1.98	45	1.26	65

ured. One would expect to observe inhibition caused in part by prompt ECX formation ("zero-time inhibition"), to an extent depending on how much substrate was present in competition for enzyme surface, plus additional inhibition caused by EC production that occurred in 20 sec.

The results (points in Fig. 4) were compared with calculations based on Eq. 13 to show only the predicted zero-time contribution (line in Fig. 4). Clearly, virtually all the inhibition observed was ascribable to a zero-time effect, so that very little EC formation occurred in 20 sec. Yet the k_2 for carbaryl is 12.2 min^{-1}, implying a half-time for conversion of ECX to EC of 3.4 sec, so that in the absence of substrate-inhibition of k_2, one would have expected a great deal of EC formation in the 20 seconds. The observed results are understandable only when one takes into account substrate inhibition of k_2, which has the effect of reducing profoundly the rate of conversion of ECX to EC.

Reactivation

The reagent 2-PAM (2-pyridinealdoxime methiodide) accelerates the k_3 step in the case of organophosphates, which in many ways act in a way parallel to the carbamates. Wilson *et al.* (3) showed that such is not the case with dimethylcarbamates. We now confirm this for the methylcarbamate carbaryl. It was found that the half-life of recovery from total inhibition was 23 min at 38°, in the presence or absence of 10^{-4} M 2-PAM. This finding is reflected in the lack of therapeutic value of 2-PAM in the poisoning of rats by carbaryl (15).

Receptor Action

In order to understand the physiological effects of carbamates, one would like to know whether they can affect cholinergic receptors as well as acetylcholinesterase. Thesleff and Quastel (16) suggest that "all acetylcholinesterase inhibitors, in sufficient concentration, have a "curarizing" action and block neuromuscular transmission." The frog rectus preparation was used, with its AChE destroyed (see Methods). Car-

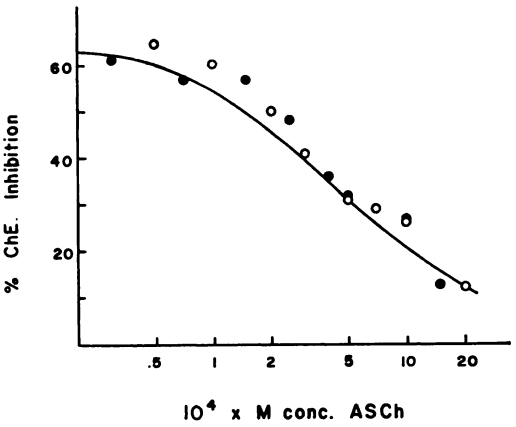

FIG. 4. *Inhibition of acetylcholinesterase (2.5 units/ml, final concentration) by carbaryl (2×10^{-4} M final concentration) mixed with various concentrations of acetylthiocholine*

Reaction was stopped after 20 sec by adding an equal volume of DTNB, 0.5 mg/ml in ethanol. Open circles and filled circles represent experiments on two different days, with different batches of reagents. The line is that calculated from Eq. 13 describing inhibition at zero time, using K_m for acetylthiocholine (1.45×10^{-4}) in place of K_s, and using $K_v = 1.01 \times 10^{-4}$ for carbaryl, from Table 1.

baryl, incubated at 10^{-4} M for 20 min, had no effect upon this preparation.

DISCUSSION

Attempts to Calculate k_1 and k_{-1}

An opportunity appears to exist to compute k_1 and k_{-1} from the data provided above, if and only if K_y is a close approximation of K_a. For then, Main analysis of short-term inhibition can be used to find K_a and k_2, Lineweaver-Burk analysis of long-term inhibition can be used to find K_m, and k_3 can be measured by observing recovery (decarbamylation) of carbamylated enzyme (2–4). One should then be able to insert these data into Eq. 6, along with the consideration that $K_a = k_{-1}/k_1$, and solve for k_1 and k_{-1}.

Calculations of k_1 and k_{-1} were indeed attempted by the above procedure. But an unfortunate aspect of Eq. 6 is that moderate errors in K_a or K_m lead to enormous variations in estimates of k_1 or k_{-1}, because the answer emerges as a small difference between large numbers. This can be seen if Eq. 6 is rearranged:

$$k_1 = \frac{k_2}{\frac{K_m}{k_3}(k_2 + k_3) - K_a} \qquad (14)$$

If one uses for carbaryl values of k_2, K_m, and (in place of K_a) K_y as given by Main kinetics (Table 1) equal to 10.1×10^{-5} along with $k_3 = 0.037$ [see O'Brien *et al.* (4)], one obtains $k_1 = 8.9 \times 10^5$. But if one uses $K_y = 12.8 \times 10^{-5}$, which is the value given by the zero-time procedure described below and is close to the value of 10.1×10^{-5} in Table 1, a negative value for k_1 is obtained. Clearly this procedure for computing k_1 and k_{-1} is applicable only if one's knowledge of all the constants is extremely precise, and such is not the case at present.

Other Interpretations of Inhibition Kinetics

The above Lineweaver-Burk interpretation contrasts with the simpler interpretation of Winteringham and Fowler (10) and Winteringham (17). They assumed that the reaction is adequately described by

$$E + CX \overset{k_a}{\rightarrow} EC \overset{k_b}{\rightarrow} E \qquad (15)$$

Using this assumption, they conclude that inhibition, expressed as v/v_0, where v is the substrate hydrolysis in an inhibited system and v_0 that in an uninhibited system, is described by

$$\frac{v}{v_0} = 1/(1 + ik_a/k_b) \qquad (16)$$

where i is inhibitor concentration. Winteringham (17) used this equation to compute the effect of dilution of whole blood cholinesterase on inhibition in a system at steady state, i.e., plateau conditions as defined above. At 25°, 10^{-5} M isopropoxyphenyl methylcarbamate gave an inhibition of 92%. Calculations based on Eq. 16 indicated that a 10-fold dilution ought to reduce the inhibition to 53%; experiment showed this value to be 76%. Similar substantial discrepancies were described for seven other conditions, and an explanation was offered involving a hypothetical nonaqueous phase into which inhibitor diffused. Direct readings from our own Lineweaver-Burk plots show that 10^{-5} M of this carbamate inhibits 92% at steady state, and 10^{-6} M inhibits 73%. Our *experimental* findings therefore agree remarkably well with Winteringham's, and this result is fully in keeping with our *theoretical* picture of the inhibition process, without requiring an additional hypothesis about a nonaqueous phase.

It was pointed out above that Reiner and Simeon-Rudolf (13) doubted that a complex (ECX) existed. The grounds were that inhibition by *o*-isopropoxyphenyl methylcarbamate was unaffected by dilution of up to 30-fold, the dilution being performed after incubating enzyme and inhibitor, and before adding substrate for the assay. This procedure is the usual one for studying inhibition; small concentrations of carbamate are employed, and the addition of substrate (at high concentration) terminates the inhibition reaction. If there were a measurable amount of ECX present and

contributing to the observed inhibition, then on dilution its level should be reduced and the observed inhibition lessened.

Reiner and Simeon-Rudolf (13) used 10^{-6} M *o*-isopropoxyphenyl methylcarbamate. If one uses K_y for this compound, i.e., 2×10^{-5} M, from our Table 1 as an approximation for K_a, then one can calculate from Eq. 7 that only 2% of the enzyme is in the complexed form immediately after the addition of carbamate; this amount declines thereafter. Furthermore, since the substrate (acetylcholine) used in the assay was 10^{-3} M, then the form measured as "inhibited" was virtually all EC, for the ECX form would be displaced competitively by the 1000-fold excess of substrate, and would not contribute to the observed inhibition. For these two reasons, one would not expect to see changes in inhibition with the dilution procedure that was employed.

Substrate Inhibition of the k_2 Step

The mechanism of this inhibition is, of course, unknown. One possibility is that carbamate-binding occurs at a different site from the carbamylation itself, and that both sites have an affinity for substrate, which can therefore affect separately the binding and carbamylation steps. The phenomenon is somewhat analogous to excess-substrate inhibition, if the latter proceeds according to Krupka (18), that is, by a second mole of acetylcholine binding to the acetylated enzyme, and thereby inhibiting the deacetylation step. Krupka provides evidence that choline and tetramethylammonium inhibit acetylcholine hydrolysis in a similar way.

A theoretical possibility would be that *all* the blockade of carbamate inhibition caused by substrate is due to an effect upon the k_2 step. However, this is clearly not the case, because the calculations of K_y based upon zero-time studies assume the occurrence of simple competition at the K_a step (as described by Eq. 13) and provide excellent agreement with K_y calculated from Main kinetics.

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